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Chromium-picolinate therapy in diabetes care: molecular and subcellular profiling revealed a necessity for individual outcome prediction, personalised treatment algorithms & new guidelines

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Abstract

Aims—Global figures clearly demonstrate inadequacy of current diabetes care: every 10 seconds one patient dies of diabetes-related pathologies. Nephropathy is the leading secondary complication of the disease. Nutritional supplement by chromium-picolinate is assumed to have beneficial therapeutic effects. However, potential toxic effects reported increase concerns about safety of chromium-picolinate. The experimental design aimed at determining, whether the treatment with clinically relevant doses of chromium-picolinate can harm through DNA damage and extensive alterations in central detoxification / cell-cycle regulating pathways in treatment of diabetes.

Methods—Well-acknowledged animal model of db/db-mice and clinically relevant doses of chromium-picolinate were used. As an index of DNA-damage, measurement of DNA-breaks was performed using “Comet Assay”-analysis. Individual and group-specific expression patterns of SOD-1 and P53 were evaluated to give a clue about central detoxification and cell-cycle regulating pathways under treatment conditions. The study was performed in a double-blind manner.

Results—Experimental data revealed highly individual reaction under treatment conditions. However, group-specific patterns were monitored: highest amount of damaged DNA - under the longest treatment with high doses, in contrast to groups with low doses of chromium-picolinate. Comet patterns were intermediate between untreated diabetized and control animals. Expression patterns demonstrated a correlation with subcellular imaging and dosage-dependent suppression under chromium-picolinate treatment.

Conclusions—This article highlights possible risks for individual long-term effects, when chromium-picolinate is used freely as a therapeutic nutritional modality agent without application of advanced diagnostic tools to predict risks and individual outcomes. Targeted measures require a creation of new guidelines for advanced Diabetes care.

Keywords

diabetes care; chromium-picolinate therapy; therapy response; subcellular imaging; molecular patterns; risk prediction; navigating diagnostic systems; individualised treatment algorithms; new guidelines; personalised medicine

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INTRODUCTION

Diabetes mellitus (DM) is a chronic medical condition involving a group of metabolic disorders of multiple aetiology. It is characterised by hyperglycaemia with disturbances in the metabolism of carbohydrate, fat and lipid metabolism resulting from defects in insulin secretion, insulin action or both. A combination of insulin resistance together with relative defect in insulin secretion, is the leading cause of DM type 2 appearance. Currently, diabetes affects around 300 million people worldwide. World Health Organisation (WHO) reports predict that the dramatic rise in DM over the next 25 years would be mainly due to type 2 diabetes (1), which is the most prevalent form of diabetes accounting for 90 % of the cases worldwide (2). In the year of 2000 as well as today, India, China and USA are the three leading countries with the highest prevalence of DM. Over the next 30 years the number of people with diabetes is expected to double (3). It is important to note that the worldwide impact of undiagnosed DM ranges between 30% and 50%. Further, there is an increased association between DM type 2 prevalence and various endogenous and exogenous factors such as socioeconomic status, age, gender, life style, physical activity, diet, urbanisation, education, occupation, smoking, environmental aspects etc. (4–8). Globally, DM is the fourth leading cause of death (9) and it is estimated that every 10 seconds a person dies from diabetes mellitus related complications (9). The increased oxidative and nitrosative stress, as a result of hyperglycemic episodes, is known to complicate even well controlled cases of diabetes, thereby resulting in micro- and macrovascular outcomes (10). The reduced bioavailability of vasodilator nitric oxide (NO) and increased NO degradation by ROS (11, 12) result in endothelial dysfunction - main initiating event leading to various chronic complications such as retinopathy, angiopathy, nephropathy, poly-neuropathy, cardiomyopathy, etc. (Figure 1). Two main contributors to chronic DM-complications are oxidative stress and subsequent activation of reactive oxygen species. Consequent DNA damage has been well documented in diabetic patients and animal models. Important is that sensitivity to oxidative stress differs among diabetics; increased sensitivity gives rise to a cascade of chronic complications appearing as “domino effect” (13–15). Secondary chronic complications in turn results in organ damage leading to shortened life span, sudden death and high mortality in the diabetics (9). DM patients are highly predisposed to cancer (16–18).

Diabetic Nephropathy (DN) is one of the most frequent and severe DM complications and the leading cause of end-stage renal disease worldwide. It requires dialysis or kidney transplantation and is reported to be an independent risk factor for cardiovascular mortalities in diabetic patients (19–21). Chromium-picolinate (CrPic), a well absorbed form of chromium has become a very popular nutritional supplement for treating type 2 diabetics and diabetes-predisposed individuals (22). It is reported that CrPic generates sales for more than 100 million dollars annually (23) and is freely available in numerous forms including pills, sport drinks, nutrition bars and chewing gums (24). CrPic is commonly supplied in the range of 200–500 µg tablets as a daily dose (25). Big advantage of chromium-picolinate is high molecule stability: the coupling of chromium with the ligand picolinate has resulted in increasing its bioavailability and high absorbing capacity compared with dietary chromium (26). However, a mechanism of chromium-picolinate action at molecular level is not completely understood. In 1999, Speetjens et al. reported that CrPic cleaves DNA: radicals in the presence of air and biological reductants, CrPic generates hydroxyl that leads to DNA damage (27). Further, organisms exposed to CrPic complexes have tendency to accumulate Cr(III) complexes intracellularly leading to genotoxic effects by formation of covalent bonds to DNA (28). Cytotoxic, genotoxic and mutagenic effects as well as activity damaging to mitochondria and induction of apoptosis have been reported for CrPic using mammalian cell cultures, *Drosophila* and animal models (29–32). Potential toxic effects reported have increased concerns about safety of CrPic. This article highlights possible risks for individual

long-term effects, when chromium-picolinate is being used freely as a therapeutic nutritional modality agent.

METHODS

Working hypothesis and experimental design

The experimental design (Figure 2) aimed at determining, whether the treatment with clinically relevant doses of chromium-picolinate can harm through DNA damage and extensive alterations in central detoxification / cell-cycle regulating pathways in treatment of diabetes. As an index of DNA-damage, measurement of DNA-breaks was carried out using “Comet Assay”-analysis. Both individual and group-specific expression patterns of SOD-1 and P53 were determined to characterize potential alterations in central detoxification and cell-cycle regulating pathways under treatment conditions. A double-blind study was performed.

Animal model

To simulate DM type 2 a well-acknowledged animal model of db/db-mice was used. Animals were grouped into eight groups with 5–6 animals in each. Group 1 represented the healthy control. Group 2 was the model mice for type 2 DM. Groups 3 – 7 were diabetes-model mice treated under individual algorithms: 5 mg/kg CrPic from 6 weeks to 6 months of age (Group 3), 10 mg/kg CrPic from 6 weeks to 6 months of age (Group 4), 100 mg/kg CrPic from 6 weeks to 6 months of age (Group 5), 100 mg/kg CrPic from 3 months to 6 months of age (Group 6), and 250 mg/kg CrPic from 3 months to 6 months of age (Group 7). Two kidney tissue samples (a and b as noted in “Experimental design”, see Figure 2) from different kidney parts, of the above groups of animals were collected and stored at –80°C till the below described analysis were performed.

Quantitative subcellular imaging by „Comet Assay”-analysis

The single cell gel electrophoresis assay (CometAssay™, Trevigen, Inc., Cat. No. 4250-050-K, USA) is a simple and effective method for evaluating DNA damage in cells. It is based upon the ability of denatured, cleaved DNA fragments to migrate out of the cell under the influence of an electric field, whereas undamaged DNA remains within the confines of the nucleoid and migrates slower. The assessment of DNA damage is done via the evaluation of the DNA “comet” tail shape and migration pattern. The cells are immobilised in a bed of low melting point agarose, on a Trevigen CometSlide™. After cell lysis, samples are treated with alkali to unwind and denature the DNA and hydrolyse sites of damage. After performing electrophoresis, staining with a fluorescent DNA intercalating dye (SYBR® Green I) is done and the sample is visualised by epifluorescence microscopy. The alkaline electrophoresis is very sensitive and detects small amounts of damage.

Cell samples were prepared immediately before starting the assay and were handled under dimmed light to prevent DNA damage from ultraviolet light. Buffers were chilled to 4°C or on ice in order to inhibit endogenous damage occurring during sample preparation. Calcium and magnesium free PBS has been used to inhibit endonuclease activities.

For the comet staining diluted SYBR® Green I was used. 100 µl of the staining solution was pipetted onto each circle of dried agarose and the slides were placed in refrigerator for 15 to 20 minutes. Afterwards the excess SYBR solution has been removed by tapping the slides. Once the slides have completely dried at room temperature in the dark, the comets were counted under epifluorescence microscopy. DNA damage was designated to four classes based on the visual aspect considering the extend of DNA migration as published earlier (33). Comets with a bright head and almost no tail were classified as class I indicating

minimal DNA damage. Whereas comets with no visible head and a long diffuse tail were classified as class IV revealing complete DNA fragmentation. Comets with intermediate characteristics were assigned to classes II and III based on the ratio $R = T/r$, in which T represents the comet's tail length and r is the radius of the comet's head. The characteristic value of R for class I is 1 and for class IV is ∞ ($r = 0$). Comet with R values ranging between $1 < R < 3$ were designated as class II.

Evaluation of specific expression patterns by „Western-blot”-analysis

All analyses were performed two times for each sample. Non-treated (control) and treated glioma cells in confluent (day 9) cultures were lysed by homogenisation in lysis buffer (9 M urea, Merck, Germany), 1 % DTT (Sigma, USA), 2 % CHAPS (Merck, Germany), 0.8 % Bio-Lyte, pH 3–10 (Bio-Rad, USA), 5 mM Pefabloc (Roche, Switzerland) followed by a centrifugation step. The protein concentration was quantified by the DC-Protein Assay (Bio-Rad, USA). Forty μ g protein of each sample were loaded onto 12 % SDS-polyacrylamide gels and electrophoresed to separate proteins. The proteins were then transferred to nitrocellulose membranes (Hybond ECL, Amersham Biosciences, UK) and afterwards incubated at room temperature in blocking-buffer (58 mM NaH_2PO_4 , 17 mM NaH_2PO_4 , 68 mM NaCl, 5 % nonfat dry milk powder; 0.1 % Tween 20) for 1 hour. Primary anti-body incubation was performed at room temperature using a 1:250 dilution of the specific anti-bodies (Santa Cruz, USA) in washing buffer I (58 mM NaH_2PO_4 , 17 mM NaH_2PO_4 , 68 mM NaCl, 1 % non-fat dry milk powder, 0.1 % Tween 20) for 1 hour. The membranes were then washed four times in the same solution. The horseradish peroxidase-labelled anti-goat secondary antibody was incubated at room temperature with the membranes in washing buffer I followed by three washes in washing buffer II (58 mM NaH_2PO_4 , 17 mM NaH_2PO_4 , 68 mM NaCl, 1 % nonfat dry milk powder; 0.3 % Tween 20) and three washes in washing buffer I. Then the membranes were reacted with chemiluminescent reagent ECL plus (Detection Kit, Amersham Biosciences, UK) for 1 hour and processed for auto-radiography. The individual signals were measured densitometrically using the „Quantity One” imaging system (Bio-Rad, USA).

Statistical Analysis

Statistical analyses were carried out using SPSS 17.0 software (SPSS, Chicago, USA). To examine the significance of differences among the groups of comparison, the univariable variance analyse Bonferroni was applied. To analyze interrelations of parameters in all groups, ANOVA (analysis of variance) test was used. The level of significance was chosen as $p < 0.05$.

RESULTS

Quantitative subcellular imaging by „Comet Assay”-analysis

Altogether around 60 thousand comets have been analysed in this study. Individual comet patterns for each animal in 7 groups of comparison were monitored. Comet assay analysis revealed highly individual comet patterns and significant differences in comets distribution among and within groups of comparison. Summarised patterns characteristic for each group of comparison are presented in Figure 3A. Corresponding amount of damaged cells is given in Figure 3B. Lowest amount of damaged cells was monitored in the control group (1). In contrast, the highest amount of damaged cells has been monitored in group 5 with longest duration of the treatment and highest dosis of CrPic for this treatment algorithm. Figure 3B will be, further, used to demonstrate correlations between subcellular („Comet Assay”) and molecular imaging („Western-blot” analysis).

Statistical analysis for corresponding distribution of comet classes in patterns of groups is demonstrated in Figure 4.

Evaluation of specific expression patterns by „Western-blot“-analysis

Molecular patterns characteristic for each group of comparison are demonstrated in Figure 5A and 5B. Corresponding subcellular imaging of damages cells (see “Comet Assay” above) are given for each group to show the correlation between molecular and subcellular levels. The lowest level of both SOD-1 and P53 was monitored in group 5 with longest duration of the treatment and highest dosis of CrPic for this treatment algorithym. The suppression of both genes correlates well with the highest level of DNA damaged in this group.

Statistical analysis was performed (Figure 6) in order to examine the significance of interrelations for protein expression differences among groups of comparison. ANOVA (analysis of variance) test was used. The level of significance was chosen at $p < 0.05$. The test showed significance of 0.531 for SOD-1 and 0.003 for p53.

DISCUSSION

Sub-cellular imaging of DNA-damage in untreated diabetic versus healthy control group

Quantitative subcellular imaging by “Comet Assay”-analysis revealed significantly higher DNA-damage in untreated diabetic group compared to healthy controls that is well in agreement with previous data published and reviewed earlier for diabetic patients [14–18]. Both comets class III and IV were representatively increased in diabetic group that together give a clue about damaged DNA. In contrast, all controls obligatory demonstrated class I comets with intact DNA.

Individuality within untreated diabetic group

Although subcellular imaging generally revealed group-specific patterns when diabetic animals were compared with controls, however, high heterogeneity was demonstrated within untreated diabetic group: in some animals intact DNA (class I comets) was monitored, whereas only comet classes with damaged DNA was monitored for others. This heterogeneity clearly demonstrates individualized reaction of organism towards diabetic condition. This is a very important observation that well corresponds with the clinic picture of diabetes [15]. Hence, we conclude that the animal model used is suitable for issue-related studies to simulate medical condition of diabetes.

Highly individual reaction of diabetized animals towards CrPic-treatments

Despite group-specific patterns, the subcellular imaging of comet assay indicated that each animal within a group responded individually to the same dosage of CrPic administered and treatment duration. Within diabetic groups, experimental animals demonstrated highly individual comet patterns with respect to single dosages and treatment algorithms. This observation is important in regard of highly individual response to the treatment algorithms applied. We conclude individual reaction of diabetized animals towards doses and duration of CrPic treatment. This observation can explain discrepancies in the literature concerning harmful effects of CrPic-therapy [25–30]. Our interpretation is an individual reaction towards CrPic-treatments unpredictable for a patient-cohort as a whole.

Group-specific comparative quantification of DNA-damage under CrPic treatment

The highest rate of apoptotic cells (class IV comets) was monitored in diabetized animals treated with the highest doses of CrPic namely in groups 5, 6 and 7. Further, the overall highest amount of damaged DNA was registered under the longest treatment with high doses

of the agent (group 5). In contrast, almost no difference in comet distribution was monitored between groups 3 and 4 with low doses of CrPic (5 mg/kg and 10 mg/kg, correspondingly) and the comet patterns were intermediate between these of untreated diabetized and control animals.

Stress- and detoxification-specific protein expression profiling in groups of comparison

SOD-1 expression patterns were comparable with exception of two groups namely untreated diabetised animals (group 2) and diabetised animals under the longest treatment with high doses of CrPic (group 5): in both groups SOD-1 expression level was significantly lower. This result demonstrates suppressed central detoxification pathway in untreated diabetes and under the longest treatment with high doses of agent and correlates well with the highest DNA damage in the treated group 5. Further, statistical analysis revealed high heterogeneity of SOD-1 expression in both groups that supplementary to sub-cellular imaging as commented above stresses an individual reaction towards diabetic condition and high-doses CrPic treatments.

P53 expression patterns demonstrated even more conclusive result in groups of comparison, namely the dosage dependent suppression of P53 under CrPic treatments. Only the administration of the lowest doses of the agent (5 mg/kg, group 3) increased P53 expression, whereas a doubled amount of CrPic applied (10 mg/kg, group 4) was the dosage high enough to suppress P53 expression. The lowest P53 expression level accompanied with the highest DNA damage was monitored in group 5. A possible interpretation of the result may be a potential genotoxic and mutagenic effect of CrPic, further pronounced under long treatment with high doses of the agent as it has been proposed by several studies carried out earlier [26–32].

Interpretation and outlook

Taken the above observations together we conclude possible risks for individual long-term effects, when chromium-picolinate is being used freely as a therapeutic nutritional modality agent without application of advanced diagnostic tools to predict individual risks and outcomes.

Definitely CrPic-therapy is only one example of several therapy forms which currently are applied “across-the-board” in diabetes care. As discussed and reviewed earlier more individualised treatment is desirable in effective protection against diabetic retinopathy and poly-neuropathy, diabetes-related cardiovascular complications and cancer [15]. Further field-related research is needed to establish simplified non-invasive diagnostic approaches for routine medical practice which would allow an accurate prediction of individualised therapy risks and outcomes. A promising technological platform has been recently created using approaches with the detection of circulating nucleic acids in blood plasma [34] and clinical proteomics of body fluids [13].

Targeted measures require a creation of new guidelines essential to regulate (renoprotective) therapy approaches and application of more individualised therapeutic modalities for advanced Diabetes care. These measures should provide a legitimate regulation for well-timed predictive diagnostics, an effective prevention and creation of individualised treatment algorithms in pre/diabetes [35].

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Figure 1.
Chronic complications associated with DM (15).

Figure 2.
Experimental design

A.

B. **Figure 3.****A.** Comet patterns characteristic for each group of comparison.**B.** Corresponding percentage of class III and IV comets representing damaged DNA in groups of comparison.

Figure 4.

A. Statistical analyses for Comets type I in groups of comparison as described in Figure 2 (“Experimental design”). Analyses were carried out using SPSS 17.0 software (SPSS, Chicago, USA) by the application of univariable variance analyse Bonferroni.

B. Statistical analyses for Comets type II in groups of comparison as described in Figure 2 (“Experimental design”). Analyses were carried out using SPSS 17.0 software (SPSS, Chicago, USA) by the application of univariable variance analyse Bonferroni.

C. Statistical analyses for Comets type III in groups of comparison as described in Figure 2 (“Experimental design”). Analyses were carried out using SPSS 17.0 software (SPSS, Chicago, USA) by the application of univariable variance analyse Bonferroni.

D. Statistical analyses for Comets type IV in groups of comparison as described in Figure 2 (“Experimental design”). Analyses were carried out using SPSS 17.0 software (SPSS, Chicago, USA) by the application of univariable variance analyse Bonferroni.

E. Statistical analyses for the ratio healthy (class I comets) *versus* damaged cells (classes II, III and IV comets) in groups of comparison as described in Figure 2 (“Experimental design”). Analyses were carried out using SPSS 17.0 software (SPSS, Chicago, USA) by the application of univariable variance analyse Bonferroni.

Corresponding levels of damaged DNA in groups of comparison (see Fig. 3B)

Corresponding levels of damaged DNA in groups of comparison (see Fig. 3B)

Figure 5.

A. Specific expression patterns of SOD-1 monitored for each group of comparison. The expression levels were analyzed by “Western-blot”. Normalized median values are represented. Expression rates of the target protein were normalized by corresponding rates of α -actin - the house keeping gene. The below graph shows percentage of corresponding amount of damaged DNA measured at subcellular level by “Comet Assay”-analysis.

B. Specific expression patterns of P53 monitored for each group of comparison. The expression levels were analyzed by “Western-blot”. Normalized median values are represented. Expression rates of the target protein were normalized by corresponding rates of α -actin, the house keeping gene. The below graph shows percentage of corresponding amount of damaged DNA measured at subcellular level by “Comet Assay”-analysis.

Figure 6.

A. Statistical analyses of expression levels of SOD-1 measured in groups of comparison as described in Figure 2 (“Experimental design”). Analyses were carried out using SPSS 17.0 software (SPSS, Chicago, USA) by the application of univariable variance analysis Bonferroni.

B. Statistical analyses of expression levels of SOD-1 measured in groups of comparison as described in Figure 2 (“Experimental design”). Analyses were carried out using SPSS 17.0 software (SPSS, Chicago, USA) by the application of univariable variance analysis Bonferroni.